

**PCT**WORLD INTELLECTUAL PROPERTY ORGANIZATION  
International Bureau

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification 5 :</b> A01H 1/00, 5/00, C12N 15/00 C12N 9/02, C12P 7/64, 1/02 C12P 1/04, 21/06, C07H 15/12 C07H 17/00	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 93/06712</b>  <b>(43) International Publication Date:</b> 15 April 1993 (15.04.93)
<b>(21) International Application Number:</b> PCT/US92/08746 <b>(22) International Filing Date:</b> 13 October 1992 (13.10.92)  <b>(30) Priority data:</b> 774,475 10 October 1991 (10.10.91) US 817,919 8 January 1992 (08.01.92) US  <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US 817,919 (CIP) Filed on 8 January 1992 (08.01.92)  <b>(71) Applicant (for all designated States except US):</b> RHONE-POULENC AGROCHIMIE [FR/FR]; 14-20, rue Pierre-Baizet, B.P. 9163, F-69263 Lyon Cédex 09 (FR).		<b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> THOMAS, Terry [US/US]; 3004 Normand, College Station, TX 77845 (US). REDDY, Avutu, S. [IN/US]; 3902 E. 29th Street, #G11, Bryan, TX 77802 (US). NUCCIO, Michael [US/US]; P.O. Box 553, College Station, TX 77841 (US). FREYSINET, Georges [FR/FR]; 21, rue de Nervieux, F-Saint-Cyr-au-Mont-d'Or (FR).  <b>(74) Agents:</b> SCOTT, Anthony, C. et al.; Scully, Scott, Murphy & Presser, 400 Garden City Plaza, Garden City, NY 11530 (US).  <b>(81) Designated States:</b> AU, BG, BR, CA, CS, HU, JP, KR, PL, RO, RU, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> PRODUCTION OF GAMMA LINOLENIC ACID BY A $\Delta 6$ -DESATURASE  <b>(57) Abstract</b>  Linoleic acid is converted into $\gamma$ -linolenic acid by the enzyme $\Delta 6$ -desaturase. The present invention is directed to an isolated nucleic acid comprising the $\Delta 6$ -desaturase gene. More particularly, the isolated nucleic acid comprises the promoter, coding region and termination regions of the $\Delta 6$ -desaturase gene. The present invention provides recombinant constructions comprising the $\Delta 6$ -desaturase coding region in functional combination with heterologous regulatory sequences. The nucleic acids and recombinant constructions of the instant invention are useful in the production of GLA in transgenic organisms.		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	LI	Liechtenstein	SK	Slovak Republic
CI	Côte d'Ivoire	LK	Sri Lanka	SN	Senegal
CM	Cameroon	LU	Luxembourg	SU	Soviet Union
CS	Czechoslovakia	MC	Monaco	TD	Chad
CZ	Czech Republic	MG	Madagascar	TG	Togo
DE	Germany	ML	Mali	UA	Ukraine
DK	Denmark	MN	Mongolia	US	United States of America
ES	Spain			VN	Viet Nam
FI	Finland				

PRODUCTION OF GAMMA LINOLENIC ACID  
BY A  $\Delta 6$ -DESATURASE

1

Linoleic acid (18:2) (LA) is transformed into  
gamma linolenic acid (18:3) (GLA) by the enzyme  $\Delta 6$ -  
5 desaturase. When this enzyme, or the nucleic acid  
encoding it, is transferred into LA-producing cells, GLA  
is produced. The present invention provides a nucleic  
acid comprising the  $\Delta 6$ -desaturase gene. More  
specifically, the nucleic acid comprises the promoter,  
10 coding region and termination regions of the  $\Delta 6$ -  
desaturase gene. The present invention is further  
directed to recombinant constructions comprising a  $\Delta 6$ -  
desaturase coding region in functional combination with  
heterologous regulatory sequences. The nucleic acids  
15 and recombinant constructions of the instant invention  
are useful in the production of GLA in transgenic  
organisms.

Unsaturated fatty acids such as linoleic  
( $C_{18}\Delta^{9,12}$ ) and  $\alpha$ -linolenic ( $C_{18}\Delta^{9,12,15}$ ) acids are  
20 essential dietary constituents that cannot be  
synthesized by vertebrates since vertebrate cells can  
introduce double bonds at the  $\Delta^9$  position of fatty acids  
but cannot introduce additional double bonds between the  
 $\Delta^9$  double bond and the methyl-terminus of the fatty acid  
25 chain. Because they are precursors of other products,  
linoleic and  $\alpha$ -linolenic acids are essential fatty  
acids, and are usually obtained from plant sources.  
Linoleic acid can be converted by mammals into  $\gamma$ -  
linolenic acid (GLA,  $C_{18}\Delta^{6,9,12}$ ) which can in turn be  
30 converted to arachidonic acid (20:4), a critically  
important fatty acid since it is an essential precursor  
of most prostaglandins.

35

-2-

1           The dietary provision of linoleic acid, by virtue  
of its resulting conversion to GLA and arachidonic acid,  
satisfies the dietary need for GLA and arachidonic acid.  
However, a relationship has been demonstrated between  
5   consumption of saturated fats and health risks such as  
hypercholesterolemia, atherosclerosis and other chemical  
disorders which correlate with susceptibility to  
coronary disease, while the consumption of unsaturated  
fats has been associated with decreased blood  
10   cholesterol concentration and reduced risk of  
atherosclerosis. The therapeutic benefits of dietary  
GLA may result from GLA being a precursor to arachidonic  
acid and thus subsequently contributing to prostaglandin  
synthesis. Accordingly, consumption of the more  
15   unsaturated GLA, rather than linoleic acid, has  
potential health benefits. However, GLA is not present  
in virtually any commercially grown crop plant.

Linoleic acid is converted into GLA by the enzyme  
 $\Delta 6$ -desaturase.  $\Delta 6$ -desaturase, an enzyme of about 359  
20   amino acids, has a membrane-bound domain and an active  
site for desaturation of fatty acids. When this enzyme  
is transferred into cells which endogenously produce  
linoleic acid but not GLA, GLA is produced. The present  
invention, by providing the gene encoding  $\Delta 6$ -desaturase,  
25   allows the production of transgenic organisms which  
contain functional  $\Delta 6$ -desaturase and which produce GLA.  
In addition to allowing production of large amounts of  
GLA, the present invention provides new dietary sources  
of GLA.

30           The present invention is directed to an isolated  
 $\Delta 6$ -desaturase gene. Specifically, the isolated gene

35

-3-

1 comprises the  $\Delta 6$ -desaturase promoter, coding region, and termination region.

The present invention is further directed to expression vectors comprising the  $\Delta 6$ -desaturase promoter, coding region and termination region.

The present invention is also directed to expression vectors comprising a  $\Delta 6$ -desaturase coding region in functional combination with heterologous regulatory regions, i.e. elements not derived from the  $\Delta 6$ -desaturase gene.

Cells and organisms comprising the vectors of the present invention, and progeny of such organisms, are also provided by the present invention.

The present invention further provides isolated bacterial  $\Delta 6$ -desaturase and is still further directed to an isolated nucleic acid encoding bacterial  $\Delta 6$ -desaturase.

The present invention further provides a method for producing plants with increased gamma linolenic acid (GLA) content which comprises transforming a plant cell with an isolated nucleic acid of the present invention and regenerating a plant with increased GLA content from said plant cell.

A method for producing chilling tolerant plants is also provided by the present invention.

Fig. 1 depicts the hydropathy profiles of the deduced amino acid sequences of Synechocystis  $\Delta 6$ -desaturase (Panel A) and  $\Delta 12$ -desaturase (Panel B). Putative membrane spanning regions are indicated by solid bars. Hydrophobic index was calculated for a window size of 19 amino acid residues [Kyte, et al. (1982) J. Molec. Biol. 157].

35

-4-

1           Fig. 2 provides gas liquid chromatography  
profiles of wild type (Panel A) and transgenic (Panel B)  
Anabaena.

5           Fig. 3 is a diagram of maps of cosmid cSy75,  
cSy13 and cSy7 with overlapping regions and subclones.  
The origins of subclones of cSy75, cSy75-3.5 and cSy7  
are indicated by the dashed diagonal lines. Restriction  
sites that have been inactivated are in parentheses.

10          Fig. 4 provides gas liquid chromatography  
profiles of wild type (Panel A) and transgenic (Panel B)  
tobacco.

          The present invention provides an isolated  
nucleic acid encoding  $\Delta 6$ -desaturase. To identify a  
nucleic acid encoding  $\Delta 6$ -desaturase, DNA is isolated  
15 from an organism which produces GLA. Said organism can  
be, for example, an animal cell, certain fungi (e.g.  
Mortierella), certain bacteria (e.g. Synechocystis) or  
certain plants (borage, Oenothera, currants). The  
isolation of genomic DNA can be accomplished by a  
20 variety of methods well-known to one of ordinary skill  
in the art, as exemplified by Sambrook et al. (1989) in  
Molecular Cloning: A Laboratory Manual, Cold Spring  
Harbor, NY. The isolated DNA is fragmented by physical  
methods or enzymatic digestion and cloned into an  
25 appropriate vector, e.g. a bacteriophage or cosmid  
vector, by any of a variety of well-known methods which  
can be found in references such as Sambrook et al.  
(1989). Expression vectors containing the DNA of the  
present invention are specifically contemplated herein.  
30 DNA encoding  $\Delta 6$ -desaturase can be identified by gain of  
function analysis. The vector containing fragmented DNA  
is transferred, for example by infection,

35

-5-

1 transconjugation, transfection, into a host organism  
that produces linoleic acid but not GLA. As used  
herein, "transformation" refers generally to the  
incorporation of foreign DNA into a host cell. Methods  
5 for introducing recombinant DNA into a host organism are  
known to one of ordinary skill in the art and can be  
found, for example, in Sambrook et al. (1989).  
Production of GLA by these organisms (i.e., gain of  
function) is assayed, for example by gas chromatography  
10 or other methods known to the ordinarily skilled  
artisan. Organisms which are induced to produce GLA,  
i.e. have gained function by the introduction of the  
vector, are identified as expressing DNA encoding  $\Delta 6$ -  
desaturase, and said DNA is recovered from the  
15 organisms. The recovered DNA can again be fragmented,  
cloned with expression vectors, and functionally  
assessed by the above procedures to define with more  
particularity the DNA encoding  $\Delta 6$ -desaturase.

As an example of the present invention, random  
20 DNA is isolated from the cyanobacteria Synechocystis  
Pasteur Culture Collection (PCC) 6803, American Type  
Culture Collection (ATCC) 27184, cloned into a cosmid  
vector, and introduced by transconjugation into the GLA-  
deficient cyanobacterium Anabaena strain PCC 7120, ATCC  
25 27893. Production of GLA from Anabaena linoleic acid is  
monitored by gas chromatography and the corresponding  
DNA fragment is isolated.

The isolated DNA is sequenced by methods well-  
known to one of ordinary skill in the art as found, for  
30 example, in Sambrook et al. (1989).

In accordance with the present invention, a DNA  
comprising a  $\Delta 6$ -desaturase gene has been isolated. More

35

-6-

1 particularly, a 3.588 kilobase (kb) DNA comprising a  $\Delta 6$ -  
desaturase gene has been isolated from the cyanobacteria  
Synechocystis. The nucleotide sequence of the 3.588 kb  
DNA was determined and is shown in SEQ ID NO:1. Open  
5 reading frames defining potential coding regions are  
present from nucleotide 317 to 1507 and from nucleotide  
2002 to 3081. To define the nucleotides responsible for  
encoding  $\Delta 6$ -desaturase, the 3.588 kb fragment that  
confers  $\Delta 6$ -desaturase activity is cleaved into two  
10 subfragments, each of which contains only one open  
reading frame. Fragment ORF1 contains nucleotides 1  
through 1704, while fragment ORF2 contains nucleotides  
1705 through 3588. Each fragment is subcloned in both  
forward and reverse orientations into a conjugal  
15 expression vector (AM542, Wolk et al. [1984] Proc. Natl.  
Acad. Sci. USA 81, 1561) that contains a cyanobacterial  
carboxylase promoter. The resulting constructs (i.e.  
ORF1(F), ORF1(R), ORF2(F) and ORF2(R)) are conjugated to  
wild-type Anabaena PCC 7120 by standard methods (see,  
20 for example, Wolk et al. (1984) Proc. Natl. Acad. Sci.  
USA 81, 1561). Conjugated cells of Anabaena are  
identified as Neo<sup>R</sup> green colonies on a brown background  
of dying non-conjugated cells after two weeks of growth  
on selective media (standard mineral media BG11N +  
25 containing 30 $\mu$ g/ml of neomycin according to Rippka et  
al., (1979) J. Gen Microbiol. 111, 1). The green  
colonies are selected and grown in selective liquid  
media (BG11N + with 15 $\mu$ g/ml neomycin). Lipids are  
extracted by standard methods (e.g. Dahmer et al.,  
30 (1989) Journal of American Oil Chemical Society 66, 543)  
from the resulting transconjugants containing the  
forward and reverse oriented ORF1 and ORF2 constructs.

35

SUBSTITUTE SHEET



-7-

- 1 For comparison, lipids are also extracted from wild-type cultures of Anabaena and Synechocystis. The fatty acid methyl esters are analyzed by gas liquid chromatography (GLC), for example with a Tracor-560 gas liquid chromatograph equipped with a hydrogen flame ionization detector and a capillary column. The results of GLC analysis are shown in Table 1.

Table 1: Occurrence of C18 fatty acids in wild-type and transgenic cyanobacteria

SOURCE	18:0	18:1	18:2	$\gamma$ 18:3	$\alpha$ 18:3	18:4
Anabaena (wild type)	+	+	+	-	+	-
Anabaena + ORF1(F)	+	+	+	-	+	-
Anabaena + ORF1(R)	+	+	+	-	+	-
Anabaena + ORF2(F)	+	+	+	+	+	+
Anabaena + ORF2(R)	+	+	+	-	+	-
Synechocystis (wild type)	+	+	+	+	-	-

- As assessed by GLC analysis, GLA deficient Anabaena gain the function of GLA production when the construct containing ORF2 in forward orientation is introduced by transconjugation. Transconjugants containing constructs with ORF2 in reverse orientation to the carboxylase promoter, or ORF1 in either orientation, show no GLA production. This analysis demonstrates that the single open reading frame (ORF2) within the 1884 bp fragment encodes  $\Delta$ 6-desaturase. The 1884 bp fragment is shown as SEQ ID NO:3. This is substantiated by the overall similarity of the hydropathy profiles between  $\Delta$ 6-desaturase and  $\Delta$ 12-

-8-

1 desaturase [Wada et al. (1990) Nature 347] as shown in  
Fig. 1 as (A) and (B), respectively.

Isolated nucleic acids encoding  $\Delta 6$ -desaturase can  
be identified from other GLA-producing organisms by the  
5 gain of function analysis described above, or by nucleic  
acid hybridization techniques using the isolated nucleic  
acid which encodes Anabaena  $\Delta 6$ -desaturase as a  
hybridization probe. Both genomic and cDNA cloning  
methods are known to the skilled artisan and are  
10 contemplated by the present invention. The  
hybridization probe can comprise the entire DNA sequence  
disclosed as SEQ. ID NO:1, or a restriction fragment or  
other DNA fragment thereof, including an oligonucleotide  
probe. Methods for cloning homologous genes by cross-  
15 hybridization are known to the ordinarily skilled  
artisan and can be found, for example, in Sambrook  
(1989) and Beltz et al. (1983) Methods in Enzymology  
100, 266.

Transgenic organisms which gain the function of  
20 GLA production by introduction of DNA encoding  $\Delta$ -  
desaturase also gain the function of octadecatetraenoic  
acid ( $18:4\Delta^{6,9,12,15}$ ) production. Octadecatetraenoic  
acid is present normally in fish oils and in some plant  
species of the Boraginaceae family (Craig et al. [1964]  
25 J. Amer. Oil Chem. Soc. 41, 209-211; Gross et al. [1976]  
Can. J. Plant Sci. 56, 659-664). In the transgenic  
organisms of the present invention, octadecatetraenoic  
acid results from further desaturation of  $\alpha$ -linolenic  
acid by  $\Delta 6$ -desaturase or desaturation of GLA by  $\Delta 15$ -  
30 desaturase.

The 359 amino acids encoded by ORF2, i.e. the  
open reading frame encoding  $\Delta 6$ -desaturase, are shown as

35

1 SEQ. ID NO:2. The present invention further  
contemplates other nucleotide sequences which encode the  
amino acids of SEQ ID NO:2. It is within the ken of the  
ordinarily skilled artisan to identify such sequences  
5 which result, for example, from the degeneracy of the  
genetic code. Furthermore, one of ordinary skill in the  
art can determine, by the gain of function analysis  
described hereinabove, smaller subfragments of the 1884  
bp fragment containing ORF2 which encode  $\Delta 6$ -desaturase.

10 The present invention contemplates any such  
polypeptide fragment of  $\Delta 6$ -desaturase and the nucleic  
acids therefor which retain activity for converting LA  
to GLA.

In another aspect of the present invention, a  
15 vector containing the 1884 bp fragment or a smaller  
fragment containing the promoter, coding sequence and  
termination region of the  $\Delta 6$ -desaturase gene is  
transferred into an organism, for example,  
cyanobacteria, in which the  $\Delta 6$ -desaturase promoter and  
20 termination regions are functional. Accordingly,  
organisms producing recombinant  $\Delta 6$ -desaturase are  
provided by this invention. Yet another aspect of this  
invention provides isolated  $\Delta 6$ -desaturase, which can be  
purified from the recombinant organisms by standard  
25 methods of protein purification. (For example, see  
Ausubel et al. [1987] Current Protocols in Molecular  
Biology, Green Publishing Associates, New York).

Vectors containing DNA encoding  $\Delta 6$ -desaturase are  
also provided by the present invention. It will be  
30 apparent to one of ordinary skill in the art that  
appropriate vectors can be constructed to direct the  
expression of the  $\Delta 6$ -desaturase coding sequence in a

-10-

1 variety of organisms. Replicable expression vectors are  
particularly preferred. Replicable expression vectors  
as described herein are DNA or RNA molecules engineered  
for controlled expression of a desired gene, i.e. the  
5  $\Delta 6$ -desaturase gene. Preferably the vectors are  
plasmids, bacteriophages, cosmids or viruses. Shuttle  
vectors, e.g. as described by Wolk *et al.* (1984) Proc.  
Natl. Acad. Sci. USA, 1561-1565 and Bustos *et al.* (1991)  
J. Bacteriol. 174, 7525-7533, are also contemplated in  
10 accordance with the present invention. Sambrook *et al.*  
(1989), Goeddel, ed. (1990) Methods in Enzymology 185  
Academic Press, and Perbal (1988) A Practical Guide to  
Molecular Cloning, John Wiley and Sons, Inc., provide  
detailed reviews of vectors into which a nucleic acid  
15 encoding the present  $\Delta 6$ -desaturase can be inserted and  
expressed. Such vectors also contain nucleic acid  
sequences which can effect expression of nucleic acids  
encoding  $\Delta 6$ -desaturase. Sequence elements capable of  
effecting expression of a gene product include  
20 promoters, enhancer elements, upstream activating  
sequences, transcription termination signals and  
polyadenylation sites. Both constitutive and tissue  
specific promoters are contemplated. For transformation  
of plant cells, the cauliflower mosaic virus (CaMV) 35S  
25 promoter and promoters which are regulated during plant  
seed maturation are of particular interest. All such  
promoter and transcriptional regulatory elements, singly  
or in combination, are contemplated for use in the  
present replicable expression vectors and are known to  
30 one of ordinary skill in the art. The CaMV 35S promoter  
is described, for example, by Restrepo *et al.* (1990)

35

-11-

1 Plant Cell 2, 987. Genetically engineered and mutated  
regulatory sequences are also contemplated.

The ordinarily skilled artisan can determine  
vectors and regulatory elements suitable for expression  
5 in a particular host cell. For example, a vector  
comprising the promoter from the gene encoding the  
carboxylase of Anabaena operably linked to the coding  
region of  $\Delta 6$ -desaturase and further operably linked to a  
termination signal from Synechocystis is appropriate for  
10 expression of  $\Delta 6$ -desaturase in cyanobacteria. "Operably  
linked" in this context means that the promoter and  
terminator sequences effectively function to regulate  
transcription. As a further example, a vector  
appropriate for expression of  $\Delta 6$ -desaturase in  
15 transgenic plants can comprise a seed-specific promoter  
sequence derived from helianthinin, napin, or glycin  
operably linked to the  $\Delta 6$ -desaturase coding region and  
further operably linked to a seed termination signal or  
the nopaline synthase termination signal.

20 In particular, the helianthinin regulatory  
elements disclosed in applicant's copending U.S.  
Application Serial No. 682,354, filed April 8, 1991 and  
incorporated herein by reference, are contemplated as  
promoter elements to direct the expression of the  $\Delta 6$ -  
25 desaturase of the present invention.

Modifications of the nucleotide sequences or  
regulatory elements disclosed herein which maintain the  
functions contemplated herein are within the scope of  
this invention. Such modifications include insertions,  
30 substitutions and deletions, and specifically  
substitutions which reflect the degeneracy of the  
genetic code.

35

SUBSTITUTE SHEET

-12-

1           Standard techniques for the construction of such  
hybrid vectors are well-known to those of ordinary skill  
in the art and can be found in references such as  
Sambrook et al. (1989), or any of the myriad of  
5   laboratory manuals on recombinant DNA technology that  
are widely available. A variety of strategies are  
available for ligating fragments of DNA, the choice of  
which depends on the nature of the termini of the DNA  
fragments. It is further contemplated in accordance  
10   with the present invention to include in the hybrid  
vectors other nucleotide sequence elements which  
facilitate cloning, expression or processing, for  
example sequences encoding signal peptides, a sequence  
encoding KDEL, which is required for retention of  
15   proteins in the endoplasmic reticulum or sequences  
encoding transit peptides which direct  $\Delta 6$ -desaturase to  
the chloroplast. Such sequences are known to one of  
ordinary skill in the art. An optimized transit peptide  
is described, for example, by Van den Broeck et al.  
20   (1985) Nature 313, 358. Prokaryotic and eukaryotic  
signal sequences are disclosed, for example, by  
Michaelis et al. (1982) Ann. Rev. Microbiol. 36, 425.

A further aspect of the instant invention  
provides organisms other than cyanobacteria which  
25   contain the DNA encoding the  $\Delta 6$ -desaturase of the  
present invention. The transgenic organisms  
contemplated in accordance with the present invention  
include bacteria, cyanobacteria, fungi, and plants and  
animals. The isolated DNA of the present invention can  
30   be introduced into the host by methods known in the art,  
for example infection, transfection, transformation or  
transconjugation. Techniques for transferring the DNA

35

SUBSTITUTE SHEET

1 of the present invention into such organisms are widely  
known and provided in references such as Sambrook et al.  
(1989).

5 A variety of plant transformation methods are  
known. The  $\Delta 6$ -desaturase gene can be introduced into  
plants by a leaf disk transformation-regeneration  
procedure as described by Horsch et al. (1985) Science  
227, 1229. Other methods of transformation, such as  
protoplast culture (Horsch et al. (1984) Science 223,  
10 496; DeBlock et al. (1984) EMBO J. 2, 2143; Barton et  
al. (1983) Cell 32, 1033) can also be used and are  
within the scope of this invention. In a preferred  
embodiment plants are transformed with Agrobacterium-  
derived vectors. However, other methods are available  
15 to insert the  $\Delta 6$ -desaturase gene of the present  
invention into plant cells. Such alternative methods  
include biolistic approaches (Klein et al. (1987) Nature  
327, 70), electroporation, chemically-induced DNA  
uptake, and use of viruses or pollen as vectors.

20 When necessary for the transformation method, the  
 $\Delta 6$ -desaturase gene of the present invention can be  
inserted into a plant transformation vector, e.g. the  
binary vector described by Bevan (1984) Nucleic Acids  
Res. 12, 8111. Plant transformation vectors can be  
25 derived by modifying the natural gene transfer system of  
Agrobacterium tumefaciens. The natural system comprises  
large Ti (tumor-inducing)-plasmids containing a large  
segment, known as T-DNA, which is transferred to  
transformed plants. Another segment of the Ti plasmid,  
30 the vir region, is responsible for T-DNA transfer. The  
T-DNA region is bordered by terminal repeats. In the  
modified binary vectors the tumor-inducing genes have

-14-

- 1 been deleted and the functions of the vir region are  
utilized to transfer foreign DNA bordered by the T-DNA  
border sequences. The T-region also contains a  
selectable marker for antibiotic resistance, and a  
5 multiple cloning site for inserting sequences for  
transfer. Such engineered strains are known as  
"disarmed" A. tumefaciens strains, and allow the  
efficient transformation of sequences bordered by the T-  
region into the nuclear genomes of plants.
- 10 Surface-sterilized leaf disks are inoculated with  
the "disarmed" foreign DNA-containing A. tumefaciens,  
cultured for two days, and then transferred to  
antibiotic-containing medium. Transformed shoots are  
selected after rooting in medium containing the  
15 appropriate antibiotic, transferred to soil and  
regenerated.

Another aspect of the present invention provides  
transgenic plants or progeny of these plants containing  
the isolated DNA of the invention. Both  
20 monocotyledenous and dicotyledenous plants are  
contemplated. Plant cells are transformed with the  
isolated DNA encoding  $\Delta 6$ -desaturase by any of the plant  
transformation methods described above. The transformed  
plant cell, usually in a callus culture or leaf disk, is  
25 regenerated into a complete transgenic plant by methods  
well-known to one of ordinary skill in the art (e.g.  
Horsch et al. (1985) Science 227, 1129). In a preferred  
embodiment, the transgenic plant is sunflower, oil seed  
rape, maize, tobacco, peanut or soybean. Since progeny  
30 of transformed plants inherit the DNA encoding  $\Delta 6$ -  
desaturase, seeds or cuttings from transformed plants  
are used to maintain the transgenic plant line.

35



-15-

1           The present invention further provides a method  
for providing transgenic plants with an increased  
content of GLA. This method includes introducing DNA  
encoding  $\Delta 6$ -desaturase into plant cells which lack or  
5 have low levels of GLA but contain LA, and regenerating  
plants with increased GLA content from the transgenic  
cells. In particular, commercially grown crop plants  
are contemplated as the transgenic organism, including,  
but not limited to, sunflower, soybean, oil seed rape,  
10 maize, peanut and tobacco.

          The present invention further provides a method  
for providing transgenic organisms which contain GLA.  
This method comprises introducing DNA encoding  $\Delta 6$ -  
desaturase into an organism which lacks or has low  
15 levels of GLA, but contains LA. In another embodiment,  
the method comprises introducing one or more expression  
vectors which comprise DNA encoding  $\Delta 12$ -desaturase and  
 $\Delta 6$ -desaturase into organisms which are deficient in both  
GLA and LA. Accordingly, organisms deficient in both LA  
20 and GLA are induced to produce LA by the expression of  
 $\Delta 12$ -desaturase, and GLA is then generated due to the  
expression of  $\Delta 6$ -desaturase. Expression vectors  
comprising DNA encoding  $\Delta 12$ -desaturase, or  $\Delta 12$ -  
desaturase and  $\Delta 6$ -desaturase, can be constructed by  
25 methods of recombinant technology known to one of  
ordinary skill in the art (Sambrook et al., 1989) and  
the published sequence of  $\Delta 12$ -desaturase (Wada et al  
[1990] Nature (London) 347, 200-203. In addition, it  
has been discovered in accordance with the present  
30 invention that nucleotides 2002-3081 of SEQ. ID NO:1  
encode cyanobacterial  $\Delta 12$ -desaturase. Accordingly, this  
sequence can be used to construct the subject expression

35

-16-

1 vectors. In particular, commercially grown crop plants  
are contemplated as the transgenic organism, including,  
but not limited to, sunflower, soybean, oil seed rape,  
maize, peanut and tobacco.

5 The present invention is further directed to a  
method of inducing chilling tolerance in plants.  
Chilling sensitivity may be due to phase transition of  
lipids in cell membranes. Phase transition temperature  
depends upon the degree of unsaturation of fatty acids  
10 in membrane lipids, and thus increasing the degree of  
unsaturation, for example by introducing  $\Delta 6$ -desaturase  
to convert LA to GLA, can induce or improve chilling  
resistance. Accordingly, the present method comprises  
introducing DNA encoding  $\Delta 6$ -desaturase into a plant  
15 cell, and regenerating a plant with improved chilling  
resistance from said transformed plant cell. In a  
preferred embodiment, the plant is a sunflower, soybean,  
oil seed rape, maize, peanut or tobacco plant.

The following examples further illustrate the  
20 present invention..

25

30

35

-17-

1

## EXAMPLE 1

## Strains and Culture Conditions

Synechocystis (PCC 6803, ATCC 27184), Anabaena (PCC 7120, ATCC 27893) and Synechococcus (PCC 7942, ATCC 33912) were grown photoautotrophically at 30°C in BG11N+ medium (Rippka et al. [1979] J. Gen. Microbiol. 111, 1-61) under illumination of incandescent lamps ( $60\mu\text{E.m}^{-2}.\text{s}^{-1}$ ). Cosmids and plasmids were selected and propagated in Escherichia coli strain DH5a on LB medium supplemented with antibiotics at standard concentrations as described by Maniatis et al. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring, New York.

15

20

25

30

35

1

## EXAMPLE 2

Construction of Synechocystis Cosmid Genomic Library

5 Total genomic DNA from Synechocystis (PCC 6803)  
was partially digested with Sau3A and fractionated on a  
sucrose gradient (Ausubel et al. [1987] Current  
10 Protocols in Molecular Biology, Greene Publishing  
Associates and Wiley Interscience, New York). Fractions  
containing 30 to 40 kb DNA fragments were selected and  
ligated into the dephosphorylated BamHI site of the  
cosmid vector, pDUCA7 (Buikema et al. [1991] J.  
15 Bacteriol. 173, 1879-1885). The ligated DNA was  
packaged in vitro as described by Ausubel et al. (1987),  
and packaged phage were propagated in E. coli DH5a  
containing the AvaI and Eco4711 methylase helper  
20 plasmid, pRL528 as described by Buikema et al. (1991).  
A total of 1152 colonies were isolated randomly and  
maintained individually in twelve 96-well microtiter  
plates.

20

25

30

35

-19-

1

## EXAMPLE 3

Gain-of-Function Expression of GLA in Anabaena

Anabaena (PCC 7120), a filamentous cyanobacterium, is deficient in GLA but contains significant amounts of linoleic acid, the precursor for GLA (Figure 2; Table 2). The Synechocystis cosmid library described in Example 2 was conjugated into Anabaena (PCC 7120) to identify transconjugants that produce GLA. Anabaena cells were grown to mid-log phase in BG11N+ liquid medium and resuspended in the same medium to a final concentration of approximately  $2 \times 10^8$  cells per ml. A mid-log phase culture of E. coli RP4 (Burkardt et al. [1979] J. Gen. Microbiol. 114, 341-348) grown in LB containing ampicillin was washed and resuspended in fresh LB medium. Anabaena and RP4 were then mixed and spread evenly on BG11N+ plates containing 5% LB. The cosmid genomic library was replica plated onto LB plates containing 50  $\mu\text{g/ml}$  kanamycin and 17.5  $\mu\text{g/ml}$  chloramphenicol and was subsequently patched onto BG11N+ plates containing Anabaena and RP4. After 24 hours of incubation at 30°C, 30  $\mu\text{g/ml}$  of neomycin was underlaid; and incubation at 30°C was continued until transconjugants appeared.

Individual transconjugants were isolated after conjugation and grown in 2 ml BG11N+ liquid medium with 15  $\mu\text{g/ml}$  neomycin. Fatty acid methyl esters were prepared from wild type cultures and cultures containing pools of ten transconjugants as follows. Wild type and transgenic cyanobacterial cultures were harvested by centrifugation and washed twice with distilled water. Fatty acid methyl esters were extracted from these cultures as described by Dahmer et al. (1989) J. Amer.

35

-20-

1 Oil. Chem. Soc. 66, 543-548 and were analyzed by Gas  
Liquid Chromatography (GLC) using a Tracor-560 equipped  
with a hydrogen flame ionization detector and capillary  
column (30 m x 0.25 mm bonded FSOT Superox II, Alltech  
5 Associates Inc., IL). Retention times and co-  
chromatography of standards (obtained from Sigma  
Chemical Co.) were used for identification of fatty  
acids. The average fatty acid composition was  
determined as the ratio of peak area of each C18 fatty  
10 acid normalized to an internal standard.

Representative GLC profiles are shown in Fig. 2.  
C18 fatty acid methyl esters are shown. Peaks were  
identified by comparing the elution times with known  
standards of fatty acid methyl esters and were confirmed  
15 by gas chromatography-mass spectrometry. Panel A  
depicts GLC analysis of fatty acids of wild type  
Anabaena. The arrow indicates the migration time of  
GLA. Panel B is a GLC profile of fatty acids of  
transconjugants of Anabaena with pAM542+1.8F. Two GLA  
20 producing pools (of 25 pools representing 250  
transconjugants) were identified that produced GLA.  
Individual transconjugants of each GLA positive pool  
were analyzed for GLA production; two independent  
transconjugants, AS13 and AS75, one from each pool, were  
25 identified which expressed significant levels of GLA and  
which contained cosmids, cSy13 and cSy75, respectively  
(Figure 3). The cosmids overlap in a region  
approximately 7.5 kb in length. A 3.5 kb NheI fragment  
of cSy75 was recloned in the vector pDUCA7 and  
30 transferred to Anabaena resulting in gain-of-function  
expression of GLA (Table 2).

35

SUBSTITUTE SHEET

-21-

1           Two NheI/Hind III subfragments (1.8 and 1.7 kb)  
of the 3.5 kb Nhe I fragment of cSy75-3.5 were subcloned  
into "pBLUESCRIPT" (Stratagene) (Figure 3) for  
sequencing. Standard molecular biology techniques were  
5 performed as described by Maniatis et al. (1982) and  
Ausubel et al. (1987). Dideoxy sequencing (Sanger et al.  
[1977] Proc. Natl. Acad. Sci. USA 74, 5463-5467) of  
pBS1.8 was performed with "SEQUENASE" (United States  
Biochemical) on both strands by using specific  
10 oligonucleotide primers synthesized by the Advanced DNA  
Technologies Laboratory (Biology Department, Texas A & M  
University). DNA sequence analysis was done with the  
GCG (Madison, WI) software as described by Devereux et  
al. (1984) Nucleic Acids Res. 12, 387-395.

15           Both NheI/HindIII subfragments were transferred  
into a conjugal expression vector, AM542, in both  
forward and reverse orientations with respect to a  
cyanobacterial carboxylase promoter and were introduced  
into Anabaena by conjugation. Transconjugants  
20 containing the 1.8 kb fragment in the forward  
orientation (AM542-1.8F) produced significant quantities  
of GLA and octadecatetraenoic acid (Figure 2; Table 2).  
Transconjugants containing other constructs, either  
reverse oriented 1.8 kb fragment or forward and reverse  
25 oriented 1.7 kb fragment, did not produce detectable  
levels of GLA (Table 2).

Figure 2 compares the C18 fatty acid profile of  
an extract from wild type Anabaena (Figure 2A) with that  
of transgenic Anabaena containing the 1.8 kb fragment of  
30 cSy75-3.5 in the forward orientation (Figure 2B). GLC  
analysis of fatty acid methyl esters from AM542-1.8F  
revealed a peak with a retention time identical to that

35

-22-

- 1 of authentic GLA standard. Analysis of this peak by gas  
chromatography-mass spectrometry (GC-MS) confirmed that  
it had the same mass fragmentation pattern as a GLA  
reference sample. Transgenic Anabaena with altered  
5 levels of polyunsaturated fatty acids were similar to  
wild type in growth rate and morphology.

10

15

20

25

30

35



-23-

1

Table 2

Composition of C18 Fatty Acids in  
Wild Type and Trasgenic Cyanobacteria

5

10

15

20

25

Strain	Fatty acid (%)					
	18:0	18:1	18:2	18:3 ( $\alpha$ )	18:3 ( $\gamma$ )	18:4
<b>Wild type</b>						
Synechocystis (sp.PCC6803)	13.6	4.5	54.5	-	27.3	-
Anabaena (sp.PCC7120)	2.9	24.8	37.1	35.2	-	-
Synechococcus (Sp.PCC7942)	20.6	79.4	-	-	-	-
<b>Anabaena Transconjugants</b>						
cSy75	3.8	24.4	22.3	9.1	27.9	12.5
cSy75-3.5	4.3	27.6	18.1	3.2	40.4	6.4
pAM542-1.8F	4.2	13.9	12.1	19.1	25.4	25.4
pAM542-1.8R	7.7	23.1	38.4	30.8	-	-
pAM542-1.7F	2.8	27.8	36.1	33.3	-	-
pAM542-1.7R	2.8	25.4	42.3	29.6	-	-
<b>Synechococcus Transformants</b>						
pAM854	27.8	72.2	-	-	-	-
pAM854- $\Delta^{12}$	4.0	43.2	46.0	-	-	-
pAM854- $\Delta^6$	18.2	81.8	-	-	-	-
pAM854- $\Delta^6$ & $\Delta^{12}$	42.7	25.3	19.5	-	16.5	-

30

18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic  
acid; 18:3( $\alpha$ ),  $\alpha$ -linolenic acid; 18:3( $\gamma$ ),  $\gamma$ -linolenic  
acid; 18:4, octadecatetraenoic acid

35

-24-

1

## EXAMPLE 4

**Transformation of Synechococcus  
with  $\Delta 6$  and  $\Delta 12$  Desaturase Genes**

5 A third cosmid, cSy7, which contains a  $\Delta 12$ -  
desaturase gene, was isolated by screening the  
Synechocystis genomic library with a oligonucleotide  
synthesized from the published Synechocystis  $\Delta 12$ -  
desaturase gene sequence (Wada et al. [1990] Nature  
(London) 347, 200-203). A 1.7 kb AvaI fragment from  
10 this cosmid containing the  $\Delta 12$ -desaturase gene was  
identified and used as a probe to demonstrate that cSy13  
not only contains a  $\Delta 6$ -desaturase gene but also a  $\Delta 12$ -  
desaturase gene (Figure 3). Genomic Southern blot  
analysis further showed that both the  $\Delta 6$ -and  $\Delta 12$ -  
15 desaturase genes are unique in the Synechocystis genome  
so that both functional genes involved in C18 fatty acid  
desaturation are linked closely in the Synechocystis  
genome.

The unicellular cyanobacterium Synechococcus (PCC  
20 7942) is deficient in both linoleic acid and GLA(3).  
The  $\Delta 12$  and  $\Delta 6$ -desaturase genes were cloned individually  
and together into pAM854 (Bustos et al. [1991] J.  
Bacteriol. 174, 7525-7533), a shuttle vector that  
contains sequences necessary for the integration of  
foreign DNA into the genome of Synechococcus (Golden et  
25 al. [1987] Methods in Enzymol. 153, 215-231).  
Synechococcus was transformed with these gene constructs  
and colonies were selected. Fatty acid methyl esters  
were extracted from transgenic Synechococcus and  
30 analyzed by GLC.

Table 2 shows that the principal fatty acids of  
wild type Synechococcus are stearic acid (18:0) and

35

SUBSTITUTE SHEET

-25-

1 oleic acid (18:1). Synechococcus transformed with  
pAM854- $\Delta$ 12 expressed linoleic acid (18:2) in addition to  
the principal fatty acids. Transformants with pAM854- $\Delta$ 6  
and  $\Delta$ 12 produced both linoleate and GLA (Table 1).  
5 These results indicated that Synechococcus containing  
both  $\Delta$ 12- and  $\Delta$ 6-desaturase genes has gained the  
capability of introducing a second double bond at the  
 $\Delta$ 12 position and a third double bond at the  $\Delta$ 6 position  
of C18 fatty acids. However, no changes in fatty acid  
10 composition was observed in the transformant containing  
pAM854- $\Delta$ 6, indicating that in the absence of substrate  
synthesized by the  $\Delta$ 12 desaturase, the  $\Delta$ 6-desaturase is  
inactive. This experiment further confirms that the 1.8  
kb NheI/HindIII fragment (Figure 3) contains both coding  
15 and promoter regions of the Synechocystis  $\Delta$ 6-desaturase  
gene. Transgenic Synechococcus with altered levels of  
polyunsaturated fatty acids were similar to wild type in  
growth rate and morphology.

20

25

30

35

-26-

1

**EXAMPLE 5****Nucleotide Sequence of  $\Delta 6$ -Desaturase**

The nucleotide sequence of the 1.8 kb fragment of cSy75-3.5 including the functional  $\Delta 6$ -desaturase gene was determined. An open reading frame encoding a polypeptide of 359 amino acids was identified (Figure 4). A Kyte-Doolittle hydropathy analysis (Kyte et al. [1982] J. Mol. Biol. 157, 105-132) identified two regions of hydrophobic amino acids that could represent transmembrane domains (Figure 1A); furthermore, the hydropathic profile of the  $\Delta 6$ -desaturase is similar to that of the  $\Delta 12$ -desaturase gene (Figure 1B; Wada et al.) and  $\Delta 9$ -desaturases (Thiede et al. [1986] J. Biol. Chem. 261, 13230-13235). However, the sequence similarity between the Synechocystis  $\Delta 6$ - and  $\Delta 12$ -desaturases is less than 40% at the nucleotide level and approximately 18% at the amino acid level.

20

25

30

35

1

## EXAMPLE 6

Transfer of Cyanobacterial  $\Delta^6$ -Desaturase into Tobacco

The cyanobacterial  $\Delta^6$ -desaturase gene was mobilized into a plant expression vector and transferred to tobacco using Agrobacterium mediated gene transfer techniques. To ensure that the transferred desaturase is appropriately expressed in leaves and developing seeds and that the desaturase gene product is targeted to the endoplasmic reticulum or the chloroplast, various expression cassettes with Synechocystis  $\Delta$ -desaturase open reading frame (ORF) were constructed. Components of these cassettes include: (i) a 35S promoter or seed specific promoter derived from the sunflower helianthinin gene to drive  $\Delta^6$ -desaturase gene expression in all plant tissues or only in developing seeds respectively, (ii) a putative signal peptide either from carrot extensin gene or sunflower helianthinin gene to target newly synthesized  $\Delta^6$ -desaturase into the ER, (iii) an ER lumen retention signal sequence (KDEL) at the COOH-terminal of the  $\Delta^6$ -desaturase ORF, and (iv) an optimized transit peptide to target  $\Delta^6$  desaturase into the chloroplast. The 35S promoter is a derivative of PRTL2 described by Restrepo et al. (1990). The optimized transit peptide sequence is described by Van de Broeck et al. (1985). The carrot extensin signal peptide is described by Chen et al (1985) EMBO J. 9, 2145.

Transgenic tobacco plants were produced containing a chimeric cyanobacterial desaturase gene, comprised of the Synechocystis  $\Delta^6$  desaturase gene fused to an endoplasmic reticulum retention sequence (KDEL) and extensin signal peptide driven by the CaMV 35S promoter. PCR amplifications of transgenic tobacco genomic DNA indicate that the  $\Delta^6$  desaturase gene was incorporated into the tobacco genome. Fatty acid methyl esters of leaves of these transgenic tobacco plants were

-28-

1 extracted and analyzed by Gas Liquid Chromatography  
(GLC). These transgenic tobacco accumulated significant  
amounts of GLA (Figure 4). Figure 4 shows fatty acid  
methyl esters as determined by GLC. Peaks were  
5 identified by comparing the elution times with known  
standards of fatty acid methyl ester. Accordingly,  
cyanobacterial genes involved in fatty acid metabolism  
can be used to generate transgenic plants with altered  
fatty acid compositions.

10

15

20

25

30

35

SUBSTITUTE SHEET

-29-

1

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

5

(i) APPLICANT: Thomas, Terry L.  
Reddy, Avutu S.  
Nuccio, Michael  
Freyssinet, Georges L.

(ii) TITLE OF INVENTION: PRODUCTION OF GAMMA LINOLENIC  
ACID BY A DELTA 6-DESATURASE

10

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:

15

(A) ADDRESSEE: Scully, Scott, Murphy & Presser  
(B) STREET: 400 Garden City Plaza  
(C) CITY: Garden City  
(D) STATE: New York  
(E) COUNTRY: United States  
(F) ZIP: 11530

(v) COMPUTER READABLE FORM:

20

(A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: IBM PC compatible  
(C) OPERATING SYSTEM: PC-DOS/MS-DOS  
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER: To be assigned  
(B) FILING DATE: 08-JAN-1992  
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

25

(A) NAME: McNulty, William E.  
(B) REGISTRATION NUMBER: 22,606  
(C) REFERENCE/DOCKET NUMBER: 8383Z

(ix) TELECOMMUNICATION INFORMATION:

30

(A) TELEPHONE: (516) 742-4343  
(B) TELEFAX: (516) 742-4366  
(C) TELEX: 230 901 SANS UR

35

-30-

1

## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3588 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

5

## (ii) MOLECULE TYPE: DNA (genomic)

## (ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 2002..3081

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

10	GCTAGCCACC AGTGACGATG CCTTGAATTT GGCCATTCTG ACCCAGGCCC GTATTCTGAA	60
	TCCCCGCATT CGCATTGTTA ATCGTTTGTT CAACCATGCC CTGGGTAAAC GTTTAGACAC	120
	CACCTTGCCA GACCACGTTA GTTTGAGTGT TTCCGCCCTG GCGGCCCCGA TTTTTTCCTT	180
	TGCGGCTTTG GGCAATCAGG CGATCGGGCA ATTGCGTTTG TTTGACCAGA CTTGGCCCCAT	240
	TCAGGAAATT GTCATTCACC AAGACCATCC CTGGCTCAAT TTACCCCTGG CGGATTTATG	300
15	GGATGATCCG AGCCGAATGT TGATCTATTA CCTACCGGCC CACAGTGAAA CGGATTTAGT	360
	AGGCGCAGTG GTGAATAATT TAACGTTGCA ATCTGGGGAC CATTTAATAG TGGGACAAAA	420
	ACCCCAACCC AAGACCAAAC GGCATCGCC TTGGCGCAA TTTTCCAAAC TGATTACCAA	480
	CCTGCGGGAG TATCAGCGGT ATGTCCAACA GGTGATATGG GTGGTGTGT TTTTATTGTT	540
	GATGATTTTT CTGGCCACCT TCATCTACGT TTCCATTGAT CAACATATTG CCCCAGTGGA	600
20	CGCGTTGTAT TTTCCGTGG GCATGATTAC CGGGCCGGT GGCAAGGAAG AGGTGGCCGA	660
	AAAGTCCCCC GATATCATCA AAGTATTCAC AGTGGTGATG ATGATCGCCG GGGCGGGGGT	720
	GATTGGTATT TGTATGCCC TACTGAATGA TTTCATCCTT GGCAGTCGCT TTAGTCAGTT	780
	TTTGATGCG GCCAAGTTAC CCGATCGCCA TCACATCATC ATTTGTGGGC TGGGGGGAGT	840
25	GAGCATGGCC ATTATTGAAG AGTTAATTCA CCAGGGCCAT GAAATTGTGG TAATCGAAAA	900
	GGATACAGAT AATCGTTTCT TGCATACGGC CCGCTCCCTG GGGGTGCCCC TAATTGTGGA	960

30

35

SUBSTITUTE SHEET



-31-

1	GGATGCCCCG CTAGAAAGAA CGTTGGCCTG CGCCAATATC AACCGAGCCG AAGCCATTGT	1020
	GGTGGCCACC AGCGACGACA CCGTTAACTT GGAAATTGGC CTAAGTGCCA AGGCGATCGC	1080
	CCCTAGCCTG CCACTGGTGT TGCGTTGCCA GGATGCCAG TTTAGCCTGT CCCTGCAGGA	1140
	AGTATTTGAA TTTGAAACGG TGCTTTGTCC GGCGBAATTG GCCACCTATT CCTTTGCGGC	1200
5	GGCGGCCCTG GGGGGCAAAA TTTTGGGCAA CGGCATGACC GATGATTTCG TGTGGGTAGC	1260
	CCTAGCCACC TTAATCACTC CTAACCATCC CTTTGCCGAC CAATTGGTTA AAATTGCAGC	1320
	CCAAAAGTCT GATTTTCGTC CCCTCTATCT AGAACGGGGT GGCAAAACCA TCCATAGCTG	1380
	GGAATTATTG GGTACCCATC TCGACTCTGG AGACGTGTTG TATTTAACCA TGCCCGCCAC	1440
10	TGCCCTAGAG CAACTTTGGC GATCGCCCCG TGCCACTGCT GATCCTCTGG ACTCTTTTTT	1500
	GGTTTAGCAT GGGGGGATGG AACTCTTGAC TCGGCCCAAT GGTGATCAAG AAAGAACGCT	1560
	TTGTCTATGT TTAGTATTTT TAAGTTAACC AACAGCAGAG GATAACTTCC AAAAGAAATT	1620
	AAGCTCAAAA AGTAGCAAAA TAAGTTTAAT TCATAACTGA GTTTTACTGC TAAACAGCGG	1680
	TGCAAAAAAG TCAGATAAAA TAAAAGCTTC ACTTCGGTTT TATATTGTGA CCATGGTTCC	1740
15	CAGGCATCTG CTCTAGGGAG TTTTCCGCT GCCTTTAGAG AGTATTTTCT CCAAGTCGGC	1800
	TAACTCCCCC ATTTTLAGGC AAAATCATAT ACAGACTATC CCAATATTGC CAGAGCTTTG	1860
	ATGACTCACT GTAGAAGGCA GACTAAAATT CTAGCAATGG ACTCCCAGTT GGAATAAATT	1920
	TTTAGTCTCC CCCGGCGCTG GAGTTTTTTT GTAGTTAATG GCGGTATAAT GTGAAAGTTT	1980
20	TTTATCTATT TAAATTATA A ATG CTA ACA GCG GAA AGA ATT AAA TTT ACC	2031
	Met Leu Thr Ala Glu Arg Ile Lys Phe Thr	
	1 5 10	
	CAG AAA CGG GGG TTT CGT CGG GTA CTA AAC CAA CGG GTG GAT GCC TAC	2079
	Gln Lys Arg Gly Phe Arg Arg Val Leu Asn Gln Arg Val Asp Ala Tyr	
	15 20 25	
	TTT GCC GAG CAT GGC CTG ACC CAA AGG GAT AAT CCC TCC ATG TAT CTG	2127
	Phe Ala Glu His Gly Leu Thr Gln Arg Asp Asn Pro Ser Met Tyr Leu	
	30 35 40	
25		
30		
35		

-32-

1	AAA ACC CTG ATT ATT GTG CTC TGG TTG TTT TCC GCT TGG GCC TTT GTG Lys Thr Leu Ile Ile Val Leu Trp Leu Phe Ser Ala Trp Ala Phe Val 45 50 55	2175
	CTT TTT GCT CCA GTT ATT TTT CCG GTG CGC CTA CTG GGT TGT ATG GTT Leu Phe Ala Pro Val Ile Phe Pro Val Arg Leu Leu Gly Cys Met Val 60 65 70	2223
5	TTG GCG ATC GCC TTG GCG GCC TTT TCC TTC AAT GTC GGC CAC GAT GCC Leu Ala Ile Ala Leu Ala Ala Phe Ser Phe Asn Val Gly His Asp Ala 75 80 85 90	2271
	AAC CAC AAT GCC TAT TCC TCC AAT CCC CAC ATC AAC CGG GTT CTG GGC Asn His Asn Ala Tyr Ser Ser Asn Pro His Ile Asn Arg Val Leu Gly 95 100 105	2319
10	ATG ACC TAC GAT TTT GTC GGG TTA TCT AGT TTT CTT TGG CGC TAT CGC Met Thr Tyr Asp Phe Val Gly Leu Ser Ser Phe Leu Trp Arg Tyr Arg 110 115 120	2367
	CAC AAC TAT TTG CAC CAC ACC TAC ACC AAT ATT CTT GGC CAT GAC GTG His Asn Tyr Leu His His Thr Tyr Thr Asn Ile Leu Gly His Asp Val 125 130 135	2415
	GAA ATC CAT GGA GAT GGC GCA GTA CGT ATG AGT CCT GAA CAA GAA CAT Glu Ile His Gly Asp Gly Ala Val Arg Met Ser Pro Glu Gln Glu His 140 145 150	2463
15	GTT GGT ATT TAT CGT TTC CAG CAA TTT TAT ATT TGG GGT TTA TAT CTT Val Gly Ile Tyr Arg Phe Gln Gln Phe Tyr Ile Trp Gly Leu Tyr Leu 155 160 165 170	2511
	TTC ATT CCC TTT TAT TGG TTT CTC TAC GAT GTC TAC CTA GTG CTT AAT Phe Ile Pro Phe Tyr Trp Phe Leu Tyr Asp Val Tyr Leu Val Leu Asn 175 180 185	2559
20	AAA GGC AAA TAT CAC GAC CAT AAA ATT CCT CCT TTC CAG CCC CTA GAA Lys Gly Lys Tyr His Asp His Lys Ile Pro Pro Phe Gln Pro Leu Glu 190 195 200	2607
	TTA GCT AGT TTG CTA GGG ATT AAG CTA TTA TGG CTC GGC TAC GTT TTC Leu Ala Ser Leu Leu Gly Ile Lys Leu Leu Trp Leu Gly Tyr Val Phe 205 210 215	2655
25	GGC TTA CCT CTG GCT CTG GGC TTT TCC ATT CCT GAA GTA TTA ATT GGT Gly Leu Pro Leu Ala Leu Gly Phe Ser Ile Pro Glu Val Leu Ile Gly 220 225 230	2703

30

35

SUBSTITUTE SHEET

-33-

1	GCT TCG GTA ACC TAT ATG ACC TAT GGC ATC GTG GTT TGC ACC ATC TTT Ala Ser Val Thr Tyr Met Thr Tyr Gly Ile Val Val Cys Thr Ile Phe 235 240 245 250	2751
	ATG CTG GCC CAT GTG TTG GAA TCA ACT GAA TTT CTC ACC CCC GAT GGT Met Leu Ala His Val Leu Glu Ser Thr Glu Phe Leu Thr Pro Asp Gly 255 260 265	2799
5	GAA TCC GGT GCC ATT GAT GAC GAG TGG GCT ATT TGC CAA ATT CGT ACC Glu Ser Gly Ala Ile Asp Asp Glu Trp Ala Ile Cys Gln Ile Arg Thr 270 275 280	2847
	ACG GCC AAT TTT GCC ACC AAT AAT CCC TTT TGG AAC TGG TTT TGT GGC Thr Ala Asn Phe Ala Thr Asn Asn Pro Phe Trp Asn Trp Phe Cys Gly 285 290 295	2895
10	GGT TTA AAT CAC CAA GTT ACC CAC CAT CTT TTC CCC AAT ATT TGT CAT Gly Leu Asn His Gln Val Thr His His Leu Phe Pro Asn Ile Cys His 300 305 310	2943
	ATT CAC TAT CCC CAA TTG GAA AAT ATT ATT AAG GAT GTT TGC CAA GAG Ile His Tyr Pro Gln Leu Glu Asn Ile Ile Lys Asp Val Cys Gln Glu 315 320 325 330	2991
15	TTT GGT GTG GAA TAT AAA GTT TAT CCC ACC TTC AAA GCG GCG ATC GCC Phe Gly Val Glu Tyr Lys Val Tyr Pro Thr Phe Lys Ala Ala Ile Ala 335 340 345	3039
	TCT AAC TAT CGC TGG CTA GAG GCC ATG GGC AAA GCA TCG TGACATTGCC Ser Asn Tyr Arg Trp Leu Glu Ala Met Gly Lys Ala Ser 350 355 360	3088
	TTGGGATTGA AGCAAAATGG CAAAATCCCT CGTAAATCTA TGATCGAAGC CTTTCTGTTG	3148
20	CCCGCCGACC AAATCCCCGA TGCTGACCAA AGGTTGATGT TGGCATTGCT CCAAACCCAC	3208
	TTTGAGGGGG TTCATTGGCC GCAGTTTCAA GCTGACCTAG GAGGCAAAGA TTGGGTGATT	3268
	TTGCTCAAAT CCGCTGGGAT ATTGAAAGGC TTCACCACCT TTGGTTTCTA CCCTGCTCAA	3328
	TGGGAAGGAC AAACCGTCAG AATTGTTTAT TCTGGTGACA CCATCACC GA CCCATCCATG	3388
	TGGTCTAACC CAGCCCTGGC CAAGGCTTGG AÇCAAGGCCA TGCAAATTCT CCACGAGGCT	3448
25	AGGCCAGAAA AATTATATTG GCTCCTGATT TCTTCCGGCT ATCGCACCTA CCGATTTTGT	3508
	AGCATTTTGT CCAAGGAATT CTATCCCCAC TATCTCCATC CCACTCCCCC GCCTGTACAA	3568

30

35

SUBSTITUTE SHEET

-34-

1 AATTTTATCC ATCAGCTAGC

3588

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 359 amino acids  
(B) TYPE: amino acid  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein.

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met Leu Thr Ala Glu Arg Ile Lys Phe Thr Gln Lys Arg Gly Phe Arg  
1 5 10 15

10 Arg Val Leu Asn Gln Arg Val Asp Ala Tyr Phe Ala Glu His Gly Leu  
20 25 30

Thr Gln Arg Asp Asn Pro Ser Met Tyr Leu Lys Thr Leu Ile Ile Val  
35 40 45

Leu Trp Leu Phe Ser Ala Trp Ala Phe Val Leu Phe Ala Pro Val Ile  
50 55 60

15 Phe Pro Val Arg Leu Leu Gly Cys Met Val Leu Ala Ile Ala Leu Ala  
65 70 75 80

Ala Phe Ser Phe Asn Val Gly His Asp Ala Asn His Asn Ala Tyr Ser  
85 90 95

Ser Asn Pro His Ile Asn Arg Val Leu Gly Met Thr Tyr Asp Phe Val  
100 105 110

20 Gly Leu Ser Ser Phe Leu Trp Arg Tyr Arg His Asn Tyr Leu His His  
115 120 125

Thr Tyr Thr Asn Ile Leu Gly His Asp Val Glu Ile His Gly Asp Gly  
130 135 140

Ala Val Arg Met Ser Pro Glu Gln Glu His Val Gly Ile Tyr Arg Phe  
145 150 155 160

25 Gln Gln Phe Tyr Ile Trp Gly Leu Tyr Leu Phe Ile Pro Phe Tyr Trp  
165 170 175

30

35

-35-

1 Phe Leu Tyr Asp Val Tyr Leu Val Leu Asn Lys Gly Lys Tyr His Asp  
180 185 190  
His Lys Ile Pro Pro Phe Gln Pro Leu Glu Leu Ala Ser Leu Leu Gly  
195 200 205  
5 Ile Lys Leu Leu Trp Leu Gly Tyr Val Phe Gly Leu Pro Leu Ala Leu  
210 215 220  
Gly Phe Ser Ile Pro Glu Val Leu Ile Gly Ala Ser Val Thr Tyr Met  
225 230 235 240  
Thr Tyr Gly Ile Val Val Cys Thr Ile Phe Met Leu Ala His Val Leu  
245 250 255  
10 Glu Ser Thr Glu Phe Leu Thr Pro Asp Gly Glu Ser Gly Ala Ile Asp  
260 265 270  
Asp Glu Trp Ala Ile Cys Gln Ile Arg Thr Thr Ala Asn Phe Ala Thr  
275 280 285  
Asn Asn Pro Phe Trp Asn Trp Phe Cys Gly Gly Leu Asn His Gln Val  
290 295 300  
Thr His His Leu Phe Pro Asn Ile Cys His Ile His Tyr Pro Gln Leu  
305 310 315 320  
15 Glu Asn Ile Ile Lys Asp Val Cys Gln Glu Phe Gly Val Glu Tyr Lys  
325 330 335  
Val Tyr Pro Thr Phe Lys Ala Ala Ile Ala Ser Asn Tyr Arg Trp Leu  
340 345 350  
Glu Ala Met Gly Lys Ala Ser  
355

20

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 1884 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: both  
(D) TOPOLOGY: linear

25

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

30

35

SUBSTITUTE SHEET

-36-

1 AGCTTCACCTT CGGTTTTATA TTGTGACCAT GGTTCCCAGG CATCTGCTCT AGGGAGTTTT 60  
TCCGCTGCCT TTAGAGAGTA TTTTCTCCAA GTCGGCTAAC TCCCCCATTT TTAGGCAAAA 120  
TCATATACAG ACTATCCCAA TATTGCCAGA GCTTTGATGA CTCACTGTAG AAGGCAGACT 180  
5 AAAATTCCTAG CAATGGACTC CCAGTTGGAA TAAATTTTTA GTCTCCCCCG GCGCTGGAGT 240  
TTTTTTGTAG TTAATGGCGG TATAATGTGA AAGTTTTTTA TCTATTTAAA TTTATAAATG 300  
CTAACAGCGG AAAGAATTAA ATTTACCCAG AAACGGGGGT TTCGTCGGGT ACTAAACCAA 360  
CGGGTGGATG CCTACTTTGC CGAGCATGGC CTGACCCAAA GGGATAATCC CTCCATGTAT 420  
CTGAAAACCC TGATTATTGT GCTCTGGTTG TTTTCCGCTT GGGCCTTTGT GCTTTTTGCT 480  
10 CCAGTTATTT TTCCGGTGCG CCTACTGGGT TGTATGGTTT TGGCGATCGC CTTGGCGGCC 540  
TTTTCTTCA ATGTCGGCCA CGATGCCAAC CACAATGCCT ATTCCTCCAA TCCCCACATC 600  
AACCGGGTTC TGGGCATGAC CTACGATTTT GTCGGGTTAT CTAGTTTTCT TTGGCGCTAT 660  
CGCCACAACCT ATTTGCACCA CACCTACACC AATATTCTTG GCCATGACGT GGAAATCCAT 720  
GGAGATGGCG CAGTACGTAT GAGTCCTGAA CAAGAACATG TTGGTATTTA TCGTTTCCAG 780  
15 CAATTTTATA TTTGGGGTTT ATATCTTTTC ATTCCCTTTT ATTGGTTTCT CTACGATGTC 840  
TACCTAGTGC TTAATAAAGG CAAATATCAC GACCATAAAA TTCCTCCTTT CCAGCCCCTA 900  
GAATTAGCTA GTTTGCTAGG GATTAAGCTA TTATGGCTCG GCTACGTTTT CGGCTTACCT 960  
CTGGCTCTGG GCTTTTCCAT TCCTGAAGTA TTAATTGGTG CTTCCGTAAC CTATATGACC 1020  
TATGGCATCG TGGTTTGACAC CATCTTTATG CTGGCCCATG TGTTGGAATC AACTGAATTT 1080  
20 CTCACCCCCG ATGGTGAATC CGGTGCCATT GATGACGAGT GGGCTATTTG CCAAATTCGT 1140  
ACCACGGCCA ATTTTGCCAC CAATAATCCC TTTTGGAACCT GGTTTTGTGG CGGTTTAAAT 1200  
CACCAAGTTA CCCACCATCT TTTCCCAAT ATTTGTCATA TTCACTATCC CCAATTGGAA 1260  
AATATTATTA AGGATGTTTG CCAAGAGTTT GGTGTGGAAT ATAAAGTTTA TCCCACCTTC 1320  
25 AAAGCGGCGA TCGCCTCTAA CTATCGCTGG CTAGAGGCCA TGGGCAAAGC ATCGTGACAT 1380  
TGCCTTGGGA TTGAAGCAAA ATGGCAAAAT CCCTCGTAAA TCTATGATCG AAGCCTTTCT 1440

30

35

SUBSTITUTE SHEET

-37-

1 GTTGCCCGCC GACCAAATCC CCGATGCTGA CCAAAGGTTG ATGTTGGCAT TGCTCCAAAC 1500  
CCACTTTGAG GGGGTTTCATT GGCCGCAGTT TCAAGCTGAC CTAGGAGGCA AAGATTGGGT 1560  
GATTTTGCTC AAATCCGCTG GGATAATTGAA AGGCTTCACC ACCTTTGGTT TCTACCCCTGC 1620  
TCAATGGGAA GGACAAACCG TCAGAATTGT TTATTCTGGT GAÇACCATCA CCGACCCATC 1680  
5 CATGTGGTCT AACCCAGCCC TGGCCAAGGC TTGGACCAAG GCCATGCAAA TTCTCCACGA 1740  
GGCTAGGCCA GAAAAATTAT ATTGGCTCCT GATTTCTTCC GGCTATCGCA CCTACCGATT 1800  
TTGAGCATT TTGCCAAGG AATTCTATCC CCACTATCTC CATCCCACTC CCCC GCCTGT 1860  
ACAAAATTTT ATCCATCAGC TAGC 1884

10

15

20

25

30

35

1 WHAT IS CLAIMED:

1. An isolated nucleic acid encoding bacterial  $\Delta 6$ -desaturase.
2. The nucleic acid of Claim 1 comprising the  
5 nucleotides of SEQ. ID NO:3.
3. An isolated nucleic acid that codes for the amino acid sequence encoded by the nucleic acid of Claim 1.
4. The isolated nucleic acid of any one of Claims 1-3 wherein said nucleic acid is contained in a vector.
- 10 5. The isolated nucleic acid of Claim 4 operably linked to a promoter and/or a termination signal capable of effecting expression of the gene product of said isolated nucleic acid.
6. The isolated nucleic acid of Claim 5 wherein said  
15 promoter is a  $\Delta 6$ -desaturase promoter, an Anabaena carboxylase promoter, a helianthinin promoter, a glycin promoter, a napin promoter, or a helianthinin tissue-specific promoter.
7. The isolated nucleic acid of Claim 5 wherein said  
20 termination signal is a Synechocystis termination signal, a nopaline synthase termination signal, or a seed termination signal.
8. The isolated nucleic acid of any one of Claims 1-7 wherein said isolated nucleic acid is contained within a transgenic organism.
- 25 9. The isolated nucleic acid of Claim 8 wherein said transgenic organism is a bacterium, a fungus, a plant cell or an animal.
10. A plant or progeny of said plant which has been regenerated from the transgenic plant cell of Claim 9.
- 30 11. The plant of Claim 10 wherein said plant is a sunflower, soybean, maize, tobacco, peanut or oil seed rape plant.



-39-

- 1           12. A method of producing a plant with increased  
gamma linolenic acid (GLA) content which comprises:  
          (a) transforming a plant cell with the isolated  
nucleic acid of any one of Claims 1-7; and  
5           (b) regenerating a plant with increased GLA content  
from said plant cell.
13. The method of Claim 12 wherein said plant is a  
sunflower, soybean, maize, tobacco, peanut or oil seed rape  
plant.
- 10           14. A method of inducing production of gamma  
linolenic acid (GLA) in an organism deficient or lacking in  
GLA with comprises transforming said organism with the  
isolated nucleic acid of any one of Claims 1-7.
15. A method of inducing production of gamma  
15 linolenic acid (GLA) in an organism deficient or lacking in  
GLA and linoleic acid (LA) which comprises transforming said  
organism with an isolated nucleic acid encoding bacterial  $\Delta 6$ -  
desaturase and an isolated nucleic acid encoding  $\Delta 12$ -  
desaturase.
- 20           16. A method of inducing production of gamma  
linolenic acid (GLA) in an organism deficient or lacking in  
GLA and linoleic acid (LA) which comprises transforming said  
organism with at least one expression vector comprising an  
isolated nucleic acid encoding bacterial  $\Delta 6$ -desaturase and an  
25 isolated nucleic acid encoding  $\Delta 12$ -desaturase.
17. The method of any one of Claims 15 or 16 wherein  
said isolated nucleic acid encoding  $\Delta 6$ -desaturase comprises  
nucleotides 317 to 1507 of SEQ. ID NO:1.
18. A method of inducing production of  
30 octadecatetraenoic acid in an organism deficient or lacking  
in gamma linolenic acid with comprises transforming said  
organism with isolated nucleic acid of any one of Claims 1-7.

35

-40-

1           19. The method of Claim 18 wherein said organism is a  
bacterium, a fungus, a plant or an animal.

          20. A method of use of the isolated nucleic acid of  
any one of Claims 1-7 to produce a plant with improved  
5 chilling resistance which comprises:

          a) transforming a plant cell with the isolated  
nucleic acid of any one of Claims 1-7; and

          b) regenerating said plant with improved chilling  
resistance from said transformed plant cell.

10           21. The method of Claim 20 wherein said plant is a  
sunflower, soybean, maize, tobacco, peanut or oil seed rape  
plant.

          22. Isolated bacterial  $\Delta 6$ -desaturase.

          23. The isolated bacterial  $\Delta 6$ -desaturase of Claim 22  
15 which has an amino acid sequence of SEQ ID NO:2.

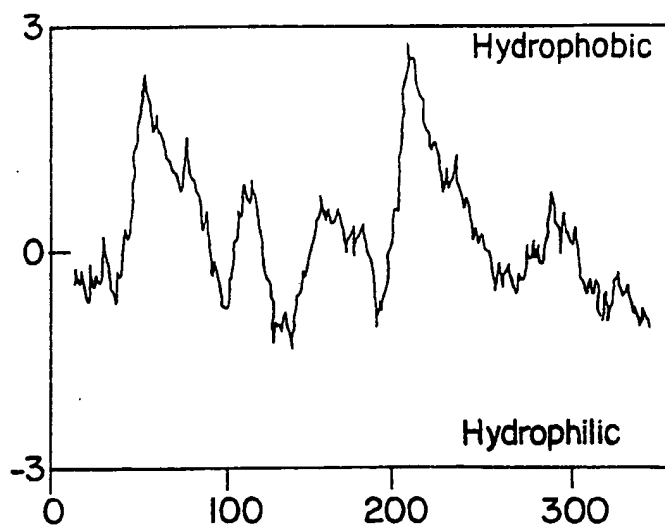
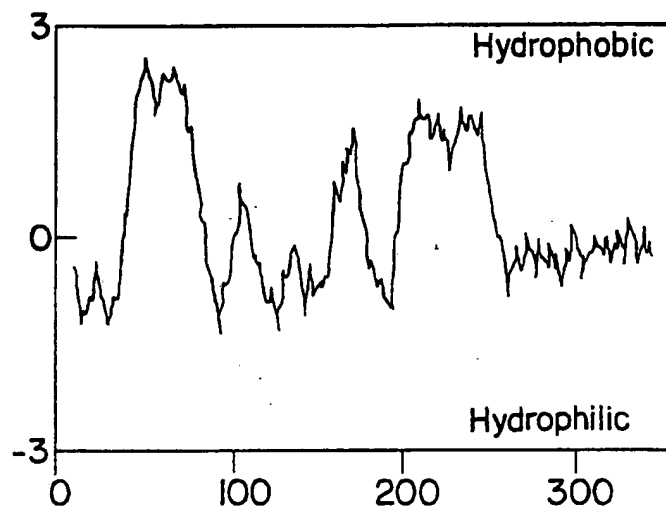
20

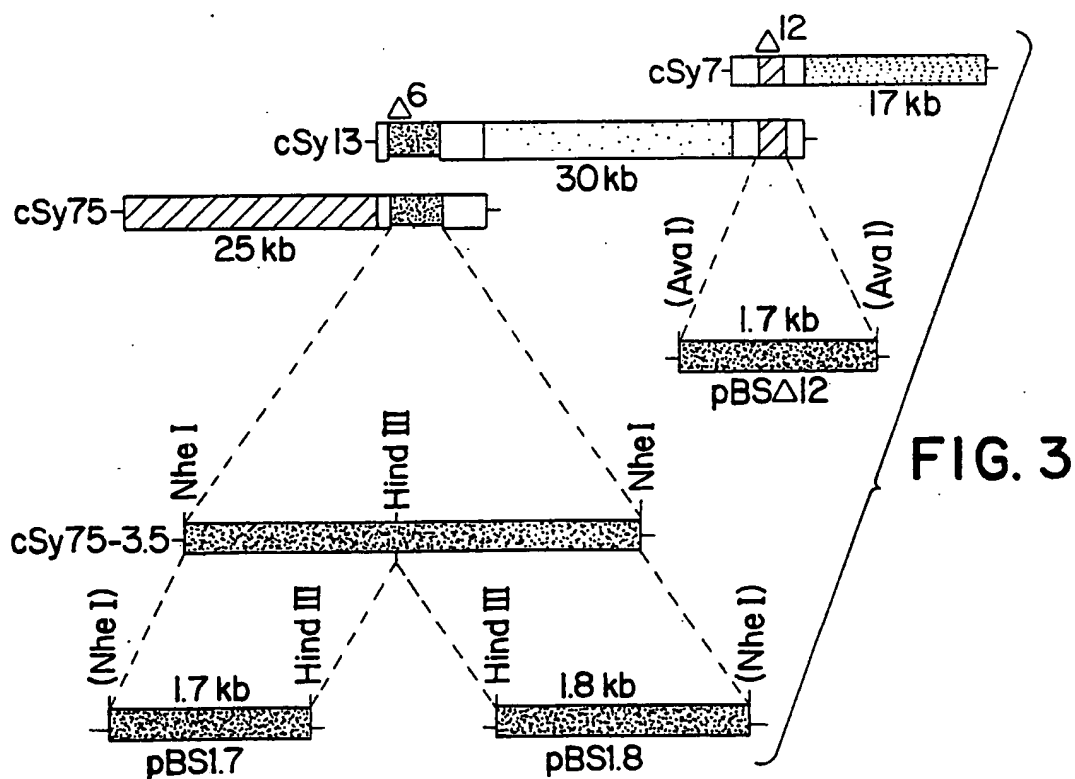
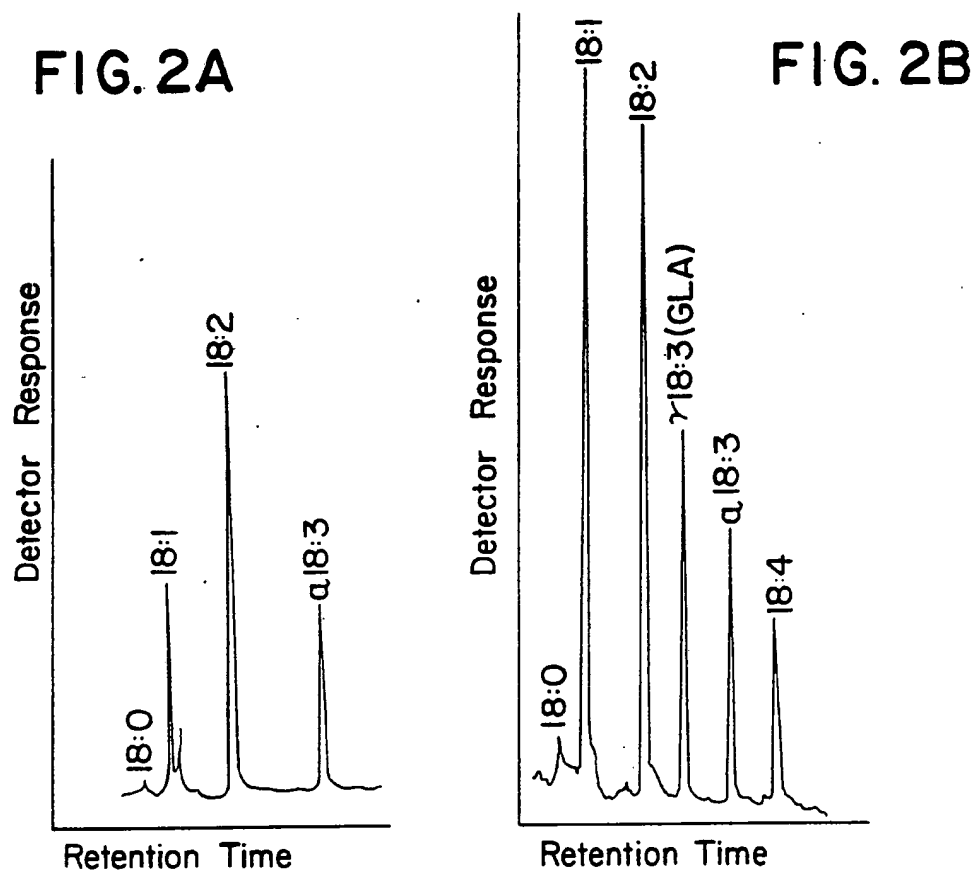
25

30

35

1 / 3





3 / 3

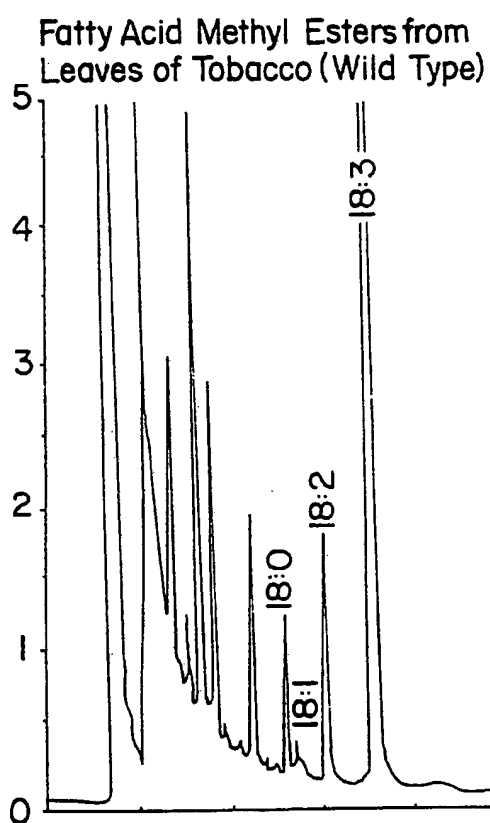


FIG. 4A

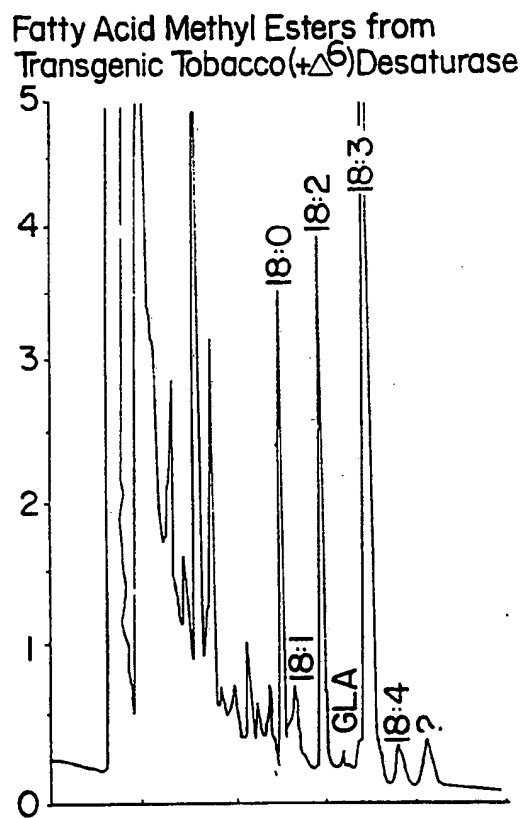


FIG. 4B

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/US92/08746

## A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) : Please See Extra Sheet.

US CL : 800/205; 435/172.3, 189, 69.1, 320.1, 134, 170, 171; 536/27

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 800/205; 435/172.3, 189, 69.1, 320.1, 134, 170, 171; 536/27;  
935/9, 30, 6, 24, 29, 38

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN/BIOSIS, CA; APS

search terms: linolenic, desaturase, delta-6, gene, DNA, cDNA,  
purif?, cyanobacteri?,

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, Volume 347, issued 13 September 1990, H. Wada et al., "Enhancement of Chilling Tolerance of a Cyanobacterium by Genetic Manipulation of Fatty Acid Desaturation", pages 200-203, especially pages 201-203.	1-23
Y	Biochemical Journal, Volume 240, issued 1986, S. Stymne et al., "Biosynthesis of $\gamma$ -Linolenic Acid in Cotyledons and Microsomal Preparations of the Developing Seeds of Common Borage ( <i>Borago officinalis</i> )", pages 385-392, especially pages 385 and 392.	1-23
Y	EP, A, 0,255, 378 (Kridl et al.) 3 February 1988, see entire document, especially columns 3-5 and 7-11.	1-23

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* "A" "E" "L" "O" "P"	Special categories of cited documents: document defining the general state of the art which is not considered to be part of particular relevance earlier document published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	"T" "X" "Y" "&"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art document member of the same patent family
--------------------------------------	--	--------------------------	--

Date of the actual completion of the international search

03 DECEMBER 1992

Date of mailing of the international search report

13 JAN 1993

Name and mailing address of the ISA/  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

Authorized officer

CHARLES C. P. RORIES, PH.D.

Facsimile No. NOT APPLICABLE

Telephone No. (703) 308-0196

# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/US92/08746

## A. CLASSIFICATION OF SUBJECT MATTER: IPC (5):

A01H 1/00, 5/00; C12N 15/00, 9/02; C12P 7/64, 1/02, 1/04, 21/06; C07H 15/12, 17/00